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INTRODUCTION:

Tumor-associated blood vessels are abnormally leaky and allow the extravasation of fibrinogen and other components of the coagulation system. Exposure of these procoagulants to extravascular matrix components and procoagulant tumor products activates the coagulation cascade and deposits large amounts of fibrin. Therefore, in tumors, angiogenic endothelial proteolytic capability would have to include fibrinolysis. We have isolated pure populations of tumor endothelial cells from xenograft breast tumors and from mammary fat pads of nude mice. RNA obtained from tumor- and mammary fat pad-associated endothelial cells was used to synthesize cDNA and cDNA libraries, which were used in differential cloning techniques to compare gene expression in tumor-associated endothelial cells with that of mammary fat pad endothelial cells. Preliminary results showed upregulation of three genes associated with fibrinolytic capability, tissue plasminogen activator (tPA), plasminogen activator inhibitor1 (PAI-1) and membrane type matrix metalloproteinase-1 (MT1-MMP), in tumor-associated endothelial cells but not in mammary fat pad endothelial cells. In these preliminary studies, urokinase plasminogen activator (uPA), its receptor (uPAR), and the tPA receptor, annexin II, were each constitutively expressed. We hypothesized that tumor endothelial cells acquire additional fibrinolytic capability during the process of angiogenesis, and this activity is important for the morphologic transformations accompanying the angiogenic process.

BODY:

1. Adaptation of the aortic ring assay to mammary fat pad vessels. Dr. Passaniti, collaborator on the project, has extensive experience with the aortic ring assay[1] using a fibrin matrix. Under his tutelage, we have adapted the assay to mammary vessels. Six to eight week old female nude mice are implanted with estrogen pellets (0.72 mg, 60 day release) to stimulate development of the mammary gland and to approximate the hormonal conditions of tumor growth. Two weeks after pellet implantation, mice are sacrificed and the mammary vessels of the inguinal mammary fat pad dissected, trimmed free of as much fat as possible, cut into small segments, and embedded in fibrin (Figure 1). If tumor cells are desired as angiogenic stimulus, they are embedded in a pellet of collagen prior to the assay, and placed in one corner of the chamber, while the mammary vessel fragment is in an opposite corner. Many cells leave the explant and invade the fibrin. Some cells form into tubes while others do not (Figure 2). We have been able to show that PECAM-1 positive cells are among the cells forming tubes. In addition, we have been able to use DiI-labeled LDL uptake to further identify endothelial tubes (Figure 3). This has been possible because of a unique fixation technique utilizing a combination of paraformaldehyde fixation with detergent and Dent's fixative, a dimethylsulfoxide/methanol protocol[2]. This is a significant advance, since successful immunostaining in fibrin matrices is rarely, if ever, reported.

Future tasks will be to perform immunostaining in the vessel assays for molecules of interest (MT1-MMP, uPA, tPA, uPAR, annexin II, etc.). We have identified antibodies that work in immunofluorescence on mouse cells for MT1-MMP (Figure 5). Over the next months, we will be ordering additional antibodies for the proteins of

interest and validating their utility using mouse EOMA cells. (EOMA cells are a murine hemangioma cell line that we use as a control for mouse endothelial cells.)

2. **Construction of sense and antisense retroviral vectors:** We proposed to abrogate MT1-MMP and tPA expression in mammary vessel assays and in animals with use of retroviral vectors to deliver antisense RNA. We still intend to use that strategy, but have first focused on cell-associated proteins rather than soluble ones. This strategy is based on the philosophy that soluble tPA may arrive in the tumor from the bloodstream, so that shutting down expression of tPA in endothelial cells may produce little effect. Therefore, we first obtained the murine cDNAs for MT1-MMP, annexin II and uPAR by long-distance RT-PCR from Swiss 3T3, EOMA, and mouse kidney total RNA using primers with Sal I restriction sites appended to the 5' ends. These cDNAs were ligated into a cloning vector (pGEM5zf(+)) and inserts in several recombinant plasmids for each cDNA were completely sequenced to identify a clone without PCR-induced errors. The plasmids with correct sequence were amplified in bacteria and digested with Sal I to liberate the cDNA. Following gel purification, cDNAs were ligated into pLNCX2 and recombinant plasmids with the inserts in the sense and antisense direction identified. This process was completed earliest for the MT1-MMP cDNA, so those plasmids were used in a test of the retroviral packaging/transduction system.

Phoenix-Eco cells[3] obtained from the ATCC through a technology transfer agreement with Dr. Garry Nolan of Stanford University were transfected with pLNCX2 without an insert, pLNCX2 with sense-oriented MT1-MMP, or pLNCX2 with antisense-oriented MT1-MMP. One-tenth concentration of a GFP-expressing plasmid, pLEGFP-N1, was included in the transfection to assess transfection efficiency (Figure 4). Following transfection, virus was collected for 24 hours and the virus-containing medium applied to EOMA cells. G418 selection of the transduced EOMA cells revealed multiple colonies of G418-resistant cells in the transduced cultures, while cultures not exposed to virus were completely killed by G418 treatment.

To test the efficacy of the antisense vector to abrogate expression of MT1-MMP, the G418 resistant cells in EOMA cultures transduced with pLNCX2-containing virus or with virus containing pLNCX2 with MT1-MMP in the antisense direction were expanded in culture and used for immunofluorescence for MT1-MMP (Figure 5). Immuno-fluorescence was performed using a mouse monoclonal antibody for MT1-MMP that recognizes the mouse protein (IM57, Oncogene Research). Careful examination of four fields per chamber revealed reduced or absent MT1-MMP immunofluorescence in cells transduced with the antisense vector, while those transduced with the vector lacking inserted sequences expressed membrane-associated MT1-MMP (Figure 5). The task is present on the statement of work in year two. These findings need to be confirmed by Western blot. However, it would appear that this vector is ready to use in the mammary vessel assays.

Our strategy of shutting down expression of various membrane receptors for uPA and tPA may not be successful. In particular, the receptor for tPA, annexin II, is a multifunctional cytoplasmic protein as well as a membrane receptor. Shutting down

expression of this protein may have toxic effects on the cell. Therefore, future tasks include construction of retroviral vectors for tPA and uPA so that antisense strategies can be employed with them also.

3. Complete PCR of existing cDNA for PAI-2 and PN-1. The preliminary results showing upregulation of tPA and MT1-MMP that formed the basis of the proposal were done on plasmid cDNA libraries synthesized from the isolated tumor-associated and mammary fat pad endothelial cells. They were done with equal amounts of plasmid DNA with a number of cycles that could be expected to maintain logarithmic amplification. In working with these plasmid libraries since then, it is apparent that the mammary fat pad endothelial cell library is not as diverse as the tumor-associated endothelial cell libraries. This is most likely due to the extremely small numbers of endothelial cells isolated from the mammary fat pads that formed the basis of the library. In order to achieve a more accurate representation of the relative expression of genes of interest in the libraries, they were standardized to GAPDH expression. Under these conditions, the differential expression of tPA and MT1-MMP was confirmed but it appears that uPA is also upregulated in tumor-associated endothelial cells. uPAR may be somewhat upregulated (Figure 6) but the degree of upregulation is slight, and could be due to variability in the PCR reaction. Moreover, one cannot consider the plasmid endothelial cell libraries to be complete representative of gene expression in the cells from which they were derived because of losses that may have occurred during library construction. The real test will come when we do these analyses on human breast cancer and reduction mammoplasty specimens, below.

PAI-1, PAI-2 and PN-1 reactions with plasmid libraries standardized to GAPDH expression have been done but results were not optimal because PCR conditions were not optimized. We are continuing these analyses.

4. Obtain slides of xenograft tumors, human breast cancer, and reduction mammoplasty, and confirm differential expression of genes of interest. Our completion of this task has been delayed by the move from Georgetown University to the University of Maryland Baltimore. At that time, we lost our collaboration with an investigator who had a patch clamp apparatus we used for microdissection. After attempts to establish a similar collaboration here failed, we set up a micromanipulator facility in our laboratory with institutional funding. This facility is now operational. In addition, the Greenebaum Cancer Center has acquired single cell laser capture microdissection capability, which is available to us. A third consideration was the state of our project to obtain large quantities of amplified RNA[4] from single microdissected endothelial cells. This project (also funded by the US Army Breast Cancer Program) is investigating ways of synthesizing long cDNAs from microdissected cells in paraffin-embedded and frozen sections in order to be able to synthesize cDNA libraries or test for the presence of multiple transcripts in material amplified from the same cell. Currently, we are able to obtain about 1000-fold amplification of cDNA synthesized from 1 pg of total RNA, and expect to be able to do better than that. In the near future, we will be applying these techniques to microdissected material. We have already been able to test

for 3 transcripts using one batch of amplified RNA from about 8 microdissected cells. These results were included in the original proposal.

When our amplified RNA protocol has been successfully applied to microdissected material and standardized, we will be able to use human samples to assay endothelial cell gene expression. There are two possibilities as to how this could be accomplished. First amplified RNA from microdissected endothelial cells could be subjected to carefully standardized PCR reactions for transcripts of interest using real-time PCR. Second, amplified RNA could be applied to a microarray containing genes of interest, including tPA, MT1-MMP, uPAR, etc. The second option gives more information but is more expensive. It also requires amplified RNA in amounts that are probably at the very upper limit of what we can produce. The decision as to which technique to use will be made based on how much amplified RNA we can produce and the cost of the microarray we need at the time we are able to use it. The Greenebaum Cancer Center has a microarray facility that makes custom arrays.

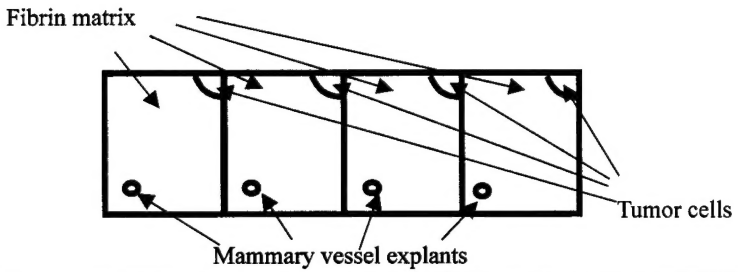

Figure 1. Cartoon depicting mammary vessel assay. Mammary vessel fragments are embedded in a fibrin or collagen gel. Endothelial sprouts invade the gel in the direction of the angiogenic stimulus (tumor cells). **Method:** Fibrinogen (3mg/ml) and amino caproic acid (0.3 mg/ml) are dissolved in DMEM 10% FBS and filtered through a 0.45 micron filter. Thrombin (100 units/ml) is added and 250 microliters placed in each chamber. Collagen pellets containing cancer cells, if desired, are positioned in the bottom layer. After the bottom layer is polymerized, the vessel fragment is positioned and 250 microliters additional fibrinogen is added and polymerized. Cultures are overlain with an additional 300 microliters of DMEM/10% FBS and incubated at 37° for approximately 8-14 days.

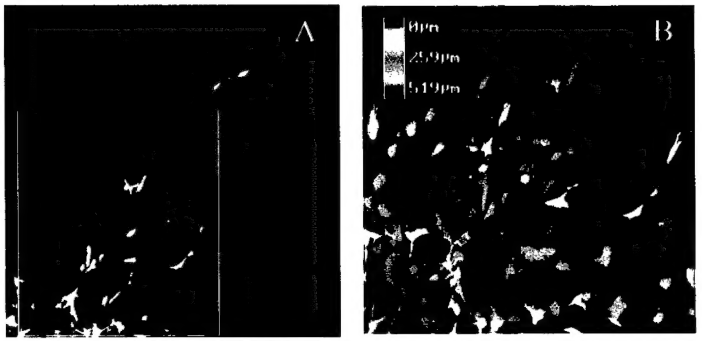

Figure 2. Confocal microscopy of mammary vessel assay. Fragments of mammary fat pad vessel were embedded in fibrin in one corner of a chamber slide (out-of-frame, lower left-hand corner) with a collagen pellet containing 10,000 tumor cells in the opposite corner (out-of-frame, upper right-hand corner). After two weeks, tubular structures have invaded the gel. **A.** Staining with Yo-Pro-1, a fluorophore that stains all cells. Branching structures are seen emanating from the explant. **B.** A 3-diminensional reconstruction of a 520 μm deep section of the same area of the gel (outlined by square in A), showing branching structures. Warmer colors are in higher focal planes.

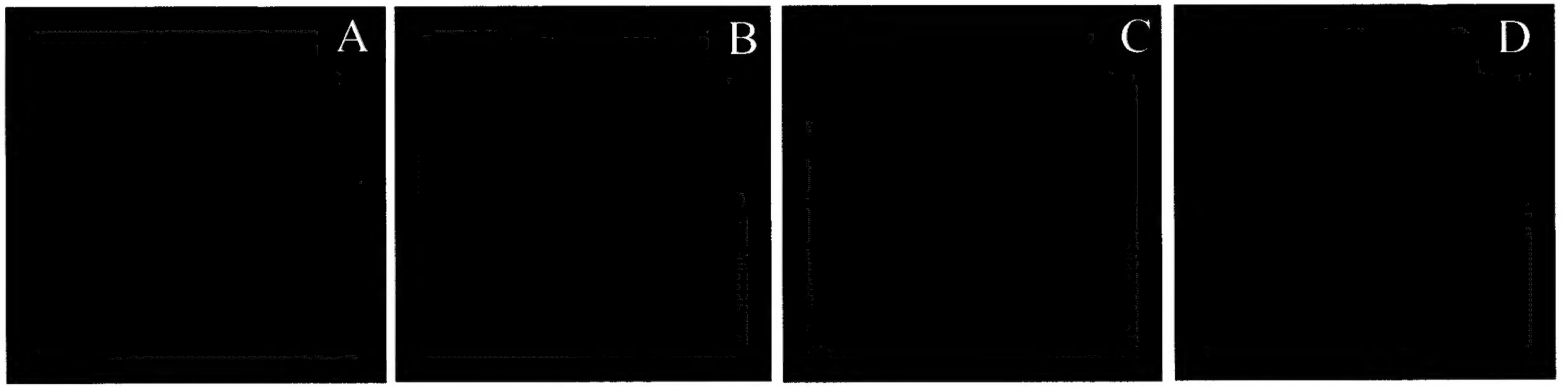

Figure 3. Confocal microscopy of mammary vessel explants in fibrin matrix. A-C. A branching structure is seen emanating from the mammary vessel explant. Green shows Yo-Pro-1 staining of nuclei, red is DiI-LDL uptake, and blue is cy-5 immunofluorescence for PECAM-1. **D** is a merged image of A-C. **Method:** Four days prior to harvest, DiI-labeled LDL (Biomedical Technologies, #BT902) is added to a concentration of 10 micrograms/ml. Fibrin cultures are fixed for 2 hours at 4° with 2% paraformaldehyde in PBS with 0.15% Triton X 100 followed by Dent's fixative (4 parts methanol and 1 part DMSO) overnight. Cultures are washed twice in absolute methanol for 20 minutes each followed by incubation for at least 1 hour in absolute methanol at -80°. After being brought to room temperature, cultures are incubated for 2 hrs in Dent's fixative plus 1 part H_2O_2, washed twice for 10 minutes each in PBS and primary rat anti-PECAM-1 antibody (PharMingin, #5533705, 5 micrograms/ml)is applied for 1 hour in 0.1% gelatin in PBS. Following three 5-minute washes with PBS, biotinylated antirat secondary antibody (Vector #4001, 5 micrograms/ml) is applied in 0.1% gelatin for 30 minutes. Following three 5-minute washes in PBS, cy-5 streptavidin (Jackson #016-170-084, 9 micrograms/ml) and Yo-Pro-1 (Molcular Probes, #Y3603, 1.3 micromolar) are applied in 0.1% gelatin in PBS. Following two 5-minute washes with PBS, the cultures are overlain with PBS for confocal microscopy.

Figure 4. Transfected ecotropic packaging cells express green fluorescent protein. A. Green fluorescence is exhibited by about 7 packaging cells in a cluster. **B.** Nomarski image of the same field, showing about 30 cells in the cluster.. **C.** Overlay of the two images. **Method:** Phoenix-Eco cells were transfected with retroviral plasmids pLNCX2 or PLNCX2 containing inserts for sense-oriented or anti-sense oriented murine MT1-MMP. PLEGFP-N1, a retroviral plasmid directing expression of GFP was included at 1/10 the concentration to determine transfection efficiency. After harvest of viral supernatants, which were used to infect murine EOMA cells, transfected packaging cells were plated in a chambered coverslip for live fluorescence imaging.

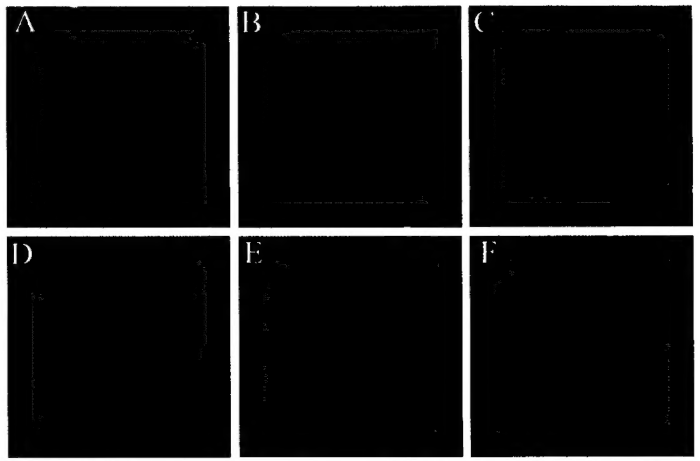

Figure 5. Transduction of EOMA cells with an antisense retroviral vector abrogates expression of MT1-MMP. In panels A, B, and C, Yo-Pro-1 staining identifies all cells. In panels D, E, and F, immunofluorescence for MT1-MMP is observed only in cells transduced with an empty retroviral vector (E) and not in cells where the primary anti-MT1-MMP antibody was omitted (D) or in cells transduced with the MT1-MMP antisense retroviral vector (F). These are representative fields of 4 per chamber (200X). **Method:** Chambered coverslips were plated with transduced EOMA cells and stained with a mouse monoclonal anti-MT1-MMP using a biotinylated secondary antibody and cy5-strepavidin disclosure of MT1-MMP immunofluorescence.

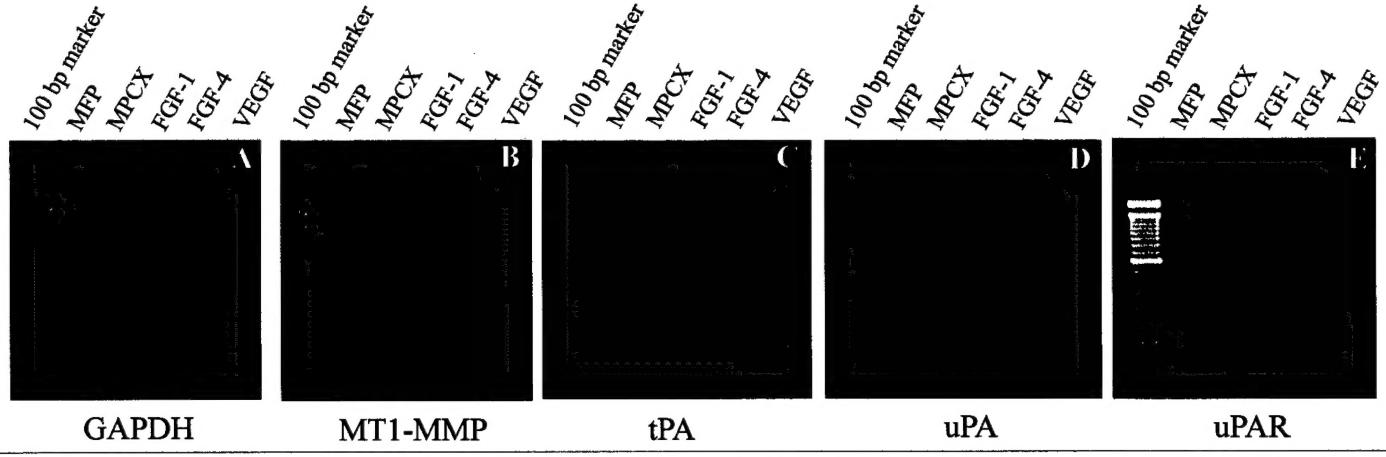

Figure 6. PCR of plasmid libraries standardized to GAPDH. PCR with specific primers for the indicated cDNA was performed on plasmid libraries isolated from tumor-associated endothelial cells. Amounts of each library used in all reactions were adjusted according to ratios of the individual libraries which gave the same intensity GAPDH band.

KEY RESEARCH OUTCOMES:

- Successful adaptation of the aortic ring assay to mammary fat pad vessels.
- Development of the ability to do immunofluorescence in fibrin matrix.
- Differential expression of MT1-MMP and tPA confirmed in existing cDNA with GAPDH control. Possible upregulation of uPA and uPAR identified.
- Establishment of micromanipulator microdissection capability at UMB.
- Construction of sense and antisense retroviral vectors for MT1-MMP, annexin II and uPAR.
- Demonstration of decreased MT1-MMP expression in EOMA cells transduced with an antisense retroviral vector.
- Transfection of packaging cells and production and titration of recombinant retrovirus.

REPORTABLE OUTCOMES:

Watson, P.A., Hannum, R.S., Emanuels, A.E., and **McLeskey, S.W.** Differential gene expression in tumor-associated endothelium. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002.

CONCLUSIONS: After the first year of funding, the techniques and reagents we need to complete the project are in hand. During the next funding year, we should be able to obtain the data we need to analyze the function of fibrinolysis in tumor-associated angiogenesis.

REFERENCE LIST:

1. Nicosia, R. F. and Tuszynski, G. P. Matrix-bound thrombospondin promotes angiogenesis in vitro. J.Cell Biol., 124: 183-193. 1994.

2. Luque, J. M., Morante-Oria, J., Riederer, B. M., and Fairen, A. Whole-mount confocal immunofluorescence of mammalian CNS. Brain Res.Brain Res.Protoc., 6: 129-133. 2001.

3. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. Production of high-titer helper-free retroviruses by transient transfection. Proc.Natl.Acad.Sci.U.S.A, 90: 8392-8396. 9-15-1993.

4. Eberwine, J. and Crino, P. Analysis of mRNA populations from single live and fixed cells of the central nervous system. Current Protocols in Neuroscience, 5.3.1-5.3.15. 1997.

APPENDIX

1. List of acronyms.

2. Abstract presented at the 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002.

ATCC	American Type Culture Collection
cDNA	copy DNA
DMEM	Dulbecco's minimal essential medium
DiI	A red fluorophore
EOMA	A mouse hemangioma cell line
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
LDL	Low density lipid
MT1-MMP	Membrane type matrix metalloproteinase 1
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PBS	Phosphate buffered saline
PN-1	Protease nexin-1
PCR	Polymerase chain reaction
RNA	Ribosenucleic acid
RT-PCR	Reverse transcriptase – polymerase chain reaction
tPA	Tissue plasminogen activator
UMB	University of Maryland at Baltimore
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

Abstract presented at the 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002

Differential Gene Expression in Tumor-Associated Endothelium

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Although tumor-associated blood vessels have unique morphology and function, little is known about expression of genes that might determine their phenotype. We have performed a one-step flow cytometric separation of endothelial cells from MCF-7 xenograft tumors growing in nude mice or from mouse mammary fat pad. RNA was immediately extracted from the sorted cells and subjected to amplified fragment length polymorphism analysis. To date, we have about 30 candidate genes that may be differentially expressed and are confirming that expression in our xenografts and in human breast cancer. These genes include members of the tissue plasminogen activator/inhibitor family, proteins involved in cell-to-cell communication and motility, and unknown ESTs. Our current results concerning differentially expressed genes will be presented.